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NANOMEDICINE

Stimuli-responsive star poly(ethylene glycol) drug conjugates for improved intracellular delivery of the drug in neuroinflammation

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ABSTRACT

N-Acetyl cysteine (NAC) is a vital drug currently under clinical trials for the treatment of neuroinflammation in maternal–fetal applications. The free sulfhydryl groups in NAC lead to high plasma protein binding, resulting in low bioavailability. Preparation and activity of conjugates of NAC with thiol terminated multi-arm (6 and 8) poly(ethylene-glycol) (PEG) with disulfide linkages involving sulfhydryls of NAC are reported. Multiple copies (5 and 7) of NAC were conjugated on 6 and 8-arm-PEG respectively. Both the conjugates released 74% of NAC within 2 h by thiol exchange reactions in the redox environment provided by glutathione (GSH) intracellularly (2–10 mM). At physiological extracellular glutathione concentration (2 μM) both the conjugates were stable and did not release NAC. MTT assay showed comparable cell viability for unmodified PEGs and both the PEG–S–S–NAC conjugates. The conjugates were readily endocytosed by cells, as confirmed by flow cytometry and confocal microscopy. Efficacy of 6 and 8-arm-PEG–S–S–NAC conjugates was evaluated on activated microglial cells (the target cells, *in vivo*) by monitoring cytokine release in lipopolysaccharide (LPS) induced inflammatory response in microglial cells using the reactive oxygen species (ROS), free radical nitrile (NO), anti-inflammatory activity and GSH depletion. The conjugates showed significant increase in antioxidant activity (more than a factor of 2) compared to free drug as seen from the inhibition of LPS induced ROS, NO, GSH and tumor necrosis factor-alpha (TNF-α) release in microglial cells.

1. Introduction

In the last few decades PEGylation of drugs has been extensively studied for drug targeting and site specific delivery [1]. PEGylation of drugs offers several advantages, including increased water solubility, plasma circulation time, improved tumor targeting by the enhanced permeability and retention (EPR) effect, and reduced immunogenic response [2,3]. PEGylation of drug involves covalent linking of the drug to polyethylene glycol (PEG) which yields the 'prodrug'. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires an enzymatic transformation within the body in order to release the active drug, and has improved delivery properties over the parent molecule [4–7].

PEG is nontoxic and it can be eliminated by a combination of renal and hepatic pathways thus making it an ideal carrier in pharmaceutical applications. PEG has the lowest level of protein or cellular absorption of any known polymer [8]. PEG has been approved for human use by FDA in dosage forms such as intravenous (IV), oral and dermal applications. PEGylation has been reported for several drugs such as PEG–Paclitaxel [9], PEG–Camptothecin [10], PEG–Ara–C [11], PEG–Doxorubicin [12], PEG–Adriamycin [13], PEG–Daunomycin [14], and anti HIV PEG–Saquinavir [15]. The examples of commercialized PEGylated products are PEG–INTRON®, PEGASYS®, ADAGEN® and ONCASPAR® and few in clinical trial include: PEG–Paclitaxel, PEG–Camptothecin and PEG–Aspartic acid [16].

Drugs are often linked to PEG *via* hydrolyzable or enzymatically cleavable bonds such as esters, carbonates, carbamates and hydrazones. In certain selective cases amide linkages which can be broken down in plasma as well as in the lysosomal compartment by peptidases or cathepsins have been explored [17]. Rapid breakdown of the conjugate can lead to dumping of the drug cargo, while too slow a release rate will compromise the efficacy of the drug. The rate of drug release is governed by the nature of the 'linker molecule', and for optimal drug release linkages should be chosen such that either pH or enzymatic degradation mediates drug release. In the case of PEG conjugates it is clear that the solubility of the prodrug will almost

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always exceed that of the original drug, usually overcoming any existing aqueous insolubility and thus increasing the possibility of more effective drug delivery.

Relatively few drugs are approved for maternal–fetal applications, due to the potential for additional side effects associated with the baby. N-acetyl cysteine (NAC) is one of the few drugs currently under clinical trials for treating neuroinflammation associated with maternal fetal infections [18,19]. However, the use of NAC requires higher and repeated dosing due to the poor bioavailability and blood stability. NAC has free sulfhydryl groups which are capable of spontaneous oxidation, and forming disulfide bonds with plasma proteins [20]. The low blood concentrations and low oral bioavailability of NAC (6–10%) can be attributed to its plasma protein binding [21,22]. The higher and frequent dosing of NAC can lead to cytotoxicity and side effects including increased blood pressure [23–28]. Hence there is a need to develop a prodrug of NAC which eliminates its plasma protein binding. Recent studies with disulfide-linked polyamidoamine (PAMAM) dendrimer–NAC conjugates showed that the conjugates released the drug effectively at intracellular glutathione levels, showing superior efficacy compared to NAC in activated microglial cells [29,30]. The disulfide bonds are sufficiently stable in the circulation and in the extracellular milieu, and are prone to rapid cleavage under a reductive environment through thiol–disulfide exchange reactions found intracellularly [31,32]. However, dendrimers are still not approved for human use, therefore, star PEG is explored in this study and is known to have longer circulation times [1]. Yet another significant advantage of PEG is that it does not invoke an immunogenic response [1]. We use a thiol terminated multi-arm-PEG scaffold (6 and 8-arm-PEG) to conjugate to NAC. The branched PEGs offer the advantage of multivalency over the linear PEGs and hence were chosen to attain higher drug payloads. The PEGylation of NAC to achieve targeted release in the treatment of neuroinflammation in perinatal applications is being explored for the first time.

The present study discusses the preparation, characterization and efficacy of disulfide-linked star PEG–NAC conjugates that are tailored to release the drug under intracellular GSH levels. The PEGylation of NAC was confirmed by ^{1}H NMR and MALDI-TOF, and the stability and the drug release from conjugates in the presence of GSH was measured using HPLC. The cellular uptake of these conjugates was assayed using flow cytometry and confocal microscopy. The antioxidative properties of the PEG–S–S–NAC conjugates (6 and 8-arm) were tested in activated BV-2 microglial cells by measuring the reactive oxygen species (ROS), free radical NO, anti-inflammatory activity and GSH depletion. To our knowledge these are the first such studies on PEG–NAC conjugates for neuroinflammation, where the conjugates show significantly better efficacy than free drug in cells.

2. Experimental procedures

2.1. Materials

The 6-arm-PEG-SH (10 kDa) was purchased from Sunbio, USA and 8-arm-PEG-SH (20 kDa) was purchased from NOF America Corporation, USA. Other reagents were obtained from assorted vendors in the highest quality available. Of these, 2, 2^1-dipyridyldisulfide (Aldrithiol), N-acetyl cysteine (NAC), glutathione (GSH), phosphate buffer saline (PBS, pH = 7.4), 2, 5 dihydroxybenzoic acid, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, monochlobimane and lipopolysaccharide (LPS), and HPLC-grade solvents were obtained from Sigma-Aldrich. Fluorescein 5-maleimide (FITC), Dulbecco's Modified Eagle Medium, fetal bovine serum, penicillin–streptomycin and 0.05% trypsin–EDTA were purchased from Invitrogen USA. The assay kits; Amplex Red hydrogen peroxide/peroxidase, nitrate/nitrite determination and Mouse TNF-α ELISA kit were purchased from Invitrogen, Cayman Chemical and BD Biosciences, respectively.

2.2. Methods

All ^{1}H NMR spectra were recorded on Varian mercury spectrometer 400 MHz in $CDCl_3$, CD_3OD. MALDI-TOF/MS spectra were recorded on a Bruker Ultraflex system equipped with a pulsed nitrogen laser (337 nm), operating in positive ion reflector mode, using 19 kV acceleration voltage and a matrix of 2, 5 dihydroxybenzoic acid.

2.2.1. Reverse phase-HPLC

HPLC characterization of conjugates was carried out with Waters HPLC instrument equipped with two pumps, an autosampler and dual UV detector interfaced to Breeze software. The mobile phase used was acetonitrile/water (pH = 2.25) both containing 0.14% TFA. The water phase was freshly prepared and both the phases were filtered and degassed prior to use. Supelco discovery BIO Wide pore C5 HPLC column (5 μm particle size, 25 cm length, 4.6 mm I.D.) equipped with two C5 supelguard cartridges (5 μm particle size, 2 cm length, 4.0 mm I.D.) was used for characterization of the conjugates as well as release and stability studies. Gradient method was used for analysis (100:0) water:acetonitrile to (60:40) water–acetonitrile in 25 min followed by returning to initial conditions in 5 min. The flow rate was 1 mL/min. The dual UV absorbance detector was used at wavelengths 210 nm and 280 nm simultaneously. Standard calibration curves were plotted for NAC, GSH and their oxidized forms (NAC–S–S–GSH, GS–SG and NAC–S–S–NAC) based on peak area obtained at 210 nm for release.

2.2.2. Stability study of conjugates

The conjugate stability was evaluated for a period of 3 days at 37 °C in a phosphate buffer saline (PBS) at physiological pH (7.4). 1 mg/mL of conjugate (1 or 3) dissolved in PBS was kept at 37 °C and stirred continuously. At specific time intervals, 20 μL of sample was withdrawn and analyzed by HPLC. All experiments were run in triplicate for statistical analysis.

2.2.3. Preparation of S-(2-thiopyridyl) N-acetyl cysteine (NAC–TP) (6)

S-(2-thiopyridyl) N-acetyl cysteine (6) was prepared by the reaction of 2, 2^1-dithiodipyridine (TP–TP) (5.398 g, 0.0245 mol) with NAC (4) (2 g, 0.0122 mol) in a mixture of methanol and water (1:1) stirred for 15 h at room temperature (r.t). Upon completion of the reaction (monitored by TLC), most of the methanol was removed *in vacuo* and the residue was dissolved in water extracted into dichloromethane and concentrated on rota-evaporator under reduced pressure to get the crude product. Crude product was purified by silica gel column chromatography by elution with dichloromethane/methanol (8:2) to pure NAC–TP (6) as a light yellow solid in 80% yield (2.66 g, 0.098 mol). Calculated mass: ESI *m/z* (M + H) 273, ^{1}H NMR (400 MHz, CD_3OD) δ, 1.99 (s, 3H), 3.10–3.20 (m, 1H), 2.30–2.38 (m, 1H), 4.65–4.70 (m, 1H) 7.20–7.27 (m, 1H, Ar), 7.80–7.85 (m, 2H Ar), 8.40–8.45 (m, 1H). ^{13}C NMR (100 MHz, CD_3OD), 21.22, 39.91, 52.05, 120.26, 121.37, 122.08, 137.98, 149.00, 159.56, 172.14.

2.2.4. Preparation of 6-arm-PEG–S–S–NAC conjugate (1)

For the preparation of 6-arm-PEG–S–S–NAC (1), NAC–TP (6) (0.245 g, 0.897 mmol) in ethanol (10 mL) was added to a solution of 6-arm-PEG–SH (7) (1.0 g, 0.1 mmol) in a PBS pH 7.4 (20 mL) and reaction was stirred at room temperature for 4 h. The reaction was monitored with HPLC. After completion of the reaction, the reaction mixture was purified by size exclusion chromatography using Sephadex LH-20 column (Amersham Pharmacia Biotech, 3.8 × 45 cm) with water as mobile phase. The fractions containing (1) were lyophilized to remove water and to get pure compound (1) in 95% yield (1.032 g, 0.0094 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ, 2.00 (s, 3H), 2.95–3.10 (m, 1H), 3.30–2.38 (m, 1H), 3.58–3.80 (br, m, 4H, –OCH_2–CH_2O–) 4.40–4.50 (m, 1H), 6.95 (br, s 1H, NH amide).